

Role of Galectin-3 in Diabetic Nephropathy

CARLA IACOBINI,* LORENA AMADIO,* GIOVANNA ODDI,* CARLO RICCI,[†]
PAOLA BARSOTTI,[‡] SERENA MISSORI,[†] MARIELLA SORCINI,*
UMBERTO DI MARIO,[†] FLAVIA PRICCI,* and GIUSEPPE PUGLIESE[†]

*Laboratory of Metabolism and Pathological Biochemistry, Section of Endocrine Biochemistry, Istituto Superiore di Sanità, Rome, Italy; and [†]Department of Clinical Sciences, Division of Endocrinology, and

[‡]Department of Experimental Medicine and Pathology, Section of Ultrastructural Pathology, "La Sapienza" University, Rome, Italy.

Abstract. The advanced glycosylation end products (AGE) participate in the pathogenesis of nephropathy and other diabetic complications through several mechanisms, including their binding to cell surface receptors. The AGE receptors include RAGE, the macrophage scavenger receptors, OST-48 (AGE-R1), 80K-H (AGE-R2), and galectin-3 (AGE-R3). Galectin-3 interacts with the β -galactoside residues of cell surface and matrix glycoproteins via the carbohydrate recognition domain and with intracellular proteins via peptide-peptide associations mediated by its N-terminus domain. These structural properties enable galectin-3 to exert multiple functions, including the mRNA splicing activity, the control of cell cycle, the regulation of cell adhesion, the modulation of allergic reactions, and the binding of AGE. The lack of transmembrane anchor sequence or signal peptide suggests that it is associated with other AGE receptors, possibly AGE-R1

and AGE-R2, to form an AGE-receptor complex, rather than playing an independent role. In target tissues of diabetic vascular complications, such as the endothelium and mesangium, galectin-3 is weakly expressed under basal conditions and is markedly upregulated by the diabetic milieu (and to a lesser extent by aging). Galectin-3-deficient mice were found to develop accelerated diabetic glomerulopathy *versus* the wild-type animals, as evidenced by the more pronounced increase in proteinuria, mesangial expansion, and matrix gene expression. This was associated with a more marked renal/glomerular AGE accumulation, suggesting that it was attributable to the lack of galectin-3 AGE-receptor function. These data indicate that galectin-3 is upregulated under diabetic conditions and is operating *in vivo* to provide protection toward AGE-induced tissue injury, as opposed to RAGE.

Diabetic nephropathy is a major cause of morbidity and mortality in patients with both type 1 and type 2 diabetes. It is characterized by predominant glomerular involvement with expansion of the mesangial region and glomerular basement membrane (GBM) thickening, associated with ialinosis of the afferent and efferent arterioles and tubulointerstitial sclerosis. The enlargement of the mesangium seems to play a major role in the progression of glomerulopathy, for it is initially accommodated by an overall growth of the glomerulus but later impinges on the glomerular capillary lumen and diminishes the filtration surface. The increase in mesangial volume is linked predominantly to increased deposition of the extracellular matrix (ECM) and possibly to an expansion of the cell compartment (1).

Hyperglycemia plays a central role in the pathogenesis of diabetic nephropathy, as shown by its prevention by strict metabolic control (2,3). The injurious effects of hyperglycemia have been attributed to its biochemical and metabolic consequences, including the increased glucose flux through the

polyol and hexosamine pathways, activation of protein kinase C, enhanced nonenzymatic glycation, and oxidative stress (1).

Advanced Glycation End Products and Their Receptors: A Major Pathway for Tissue Injury in Diabetes and Other Diseases

The advanced glycosylation end products (AGE) are heterogeneous compounds that accumulate in diabetes as a result of several mechanisms, including increased carbohydrate and lipid substrate availability, oxidative conditions favoring the glycation process, intake from dietary sources and tobacco smoke, impaired detoxification, and, in the case of associated renal failure, reduced kidney clearance (1).

In addition to displaying direct, physicochemical effects, such as trapping and cross-linking of macromolecules, AGE exert indirect, biologic effects, mediated by cell surface receptors. The binding of AGE to these receptors and the downstream signaling and transcriptional events seem to play a pivotal role in modulating target tissue injury in diabetes. In fact, the AGE receptor-mediated pathway has a dual function, because it is involved in AGE-induced cell activation, that results in dysregulated tissue remodeling but also in the removal of irreversibly glycated molecules, which is a major mechanism for protecting tissues from damaging AGE (4).

Several AGE-binding proteins have been identified so far, including the 35-kD member of the Ig superfamily called RAGE (5); the macrophage scavenger receptors classes A (6)

Correspondence to Dr. Giuseppe Pugliese, Dipartimento di Scienze Cliniche (Endocrinologia), Viale del Policlinico, 155-00161 Rome, Italy. Phone: +39-064957236; Fax: +39-0649972619; E-mail: giuseppe.pugliese@uniroma1.it
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and B (7); the 60-kD protein OST-48, or AGE-R1 (8); the 90-kD protein 80K-H, or AGE-R2 (8); and the 32-kD protein galectin-3, or AGE-R3 (9). This redundancy could suggest binding and/or functional specificity of AGE receptors; alternatively, not all of these receptors might be relevant to AGE binding *in vivo* (10). At present, RAGE seems to be involved in cell activation (5) and the macrophage scavenger receptors in AGE degradation (6,7). Conversely, the function of AGE-R1, AGE-R2, and, particularly, AGE-R3 is still largely unknown, although it seems more likely that they behave as an AGE-receptor complex rather than as individual receptor molecules (9).

AGE receptors differ from classical hormone and cytokine receptors not only for their heterogeneity. In fact, they are multifunctional receptors, as a result of their ability to interact with several other molecules or classes of molecules cross-competing for binding (multiligand receptors). For this reason, they are implicated in several pathophysiologic conditions, in addition to diabetes and aging. Moreover, they are not down-regulated but rather upregulated by their ligands, with consequent propagation and amplification of effects. Thus, in the presence of injurious stimuli causing persistent ligand accumulation, this represents a major mechanism for disease maintenance and progression. These features are typical but not unique of RAGE, because they apply also to other AGE receptors, particularly galectin-3, that is the focus of this review.

Galectin-3: Structure–Function Relationships for a Versatile Molecule

Galectins belong to the lectin family of carbohydrate-binding proteins. They share two key characteristics: lactose/galactose-binding affinity and highly conserved residues in the carbohydrate recognition domain (CRD) among members of the family. Actually, the CRD contains a more conserved core binding site for galactose (with lower affinity) and a less conserved extended binding site for larger oligosaccharides (with higher affinity). Thus, while conserving their ability to recognize basic disaccharide units, galectins have evolved their sugar-binding specificity by enhancing affinity to either branched, repeated, or substituted glycans, as shown in a recent analysis (11).

The structure of galectin-3 differs from that of the other galectins for the unique proline-glycine-alanine-tyrosine-rich repeat motif fused onto the CRD; for this reason, it is the sole component of the “chimera” group, whereas the other galectins belong to the “prototype” group or the “tandem repeat” group, depending on the presence of one or two CRD, respectively (12).

Galectin-3 expression in tissues seems to be developmentally regulated, being more abundant during embryogenesis and development than in the adult life, when it is detected in various epithelial cells and cartilage as well as inflammatory cells (12). Galectin-3 shows a ubiquitous localization within the cell and is also secreted into the extracellular space, although it lacks a signal sequence for transfer into the endo-

plasmic reticulum and Golgi compartments and entry into classical secretory pathways (13). Its intracellular distribution depends on the proliferation state of the cell, being mainly cytoplasmic in quiescent cells and nuclear in replicating cells (12). Phosphorylation at N-terminal serine 6 (and, to a lesser extent, 12) by kinase enzymes is a fundamental event for galectin-3 shuttling from the nucleus to the cytoplasm, where only the phosphorylated form can be detected (12). Phosphorylation is important also for galectin-3 activity. It is interesting that serine phosphorylation inhibits galectin-3 adhesion to matrix (14), whereas it favors its antiapoptotic function (15), as shown by experiments using site-directed mutagenesis of galectin-3.

The dual localization of galectin-3 within the cell determines two different modes of interaction with proteins. Extracellular galectin-3 interacts with the β -galactoside residues of several ECM and cell surface glycoproteins via the CRD; this is the classical lectin-glycoconjugate interaction. Conversely, interactions of intracellular galectin-3 occur via peptide–peptide associations mediated by its N-terminus domain, with the exception of binding to cytokeratins. When bound to glycoconjugates, galectin-3 is also capable of peptide–peptide homodimeric association, whereas, in the absence of these ligands, it self-associates in a C-terminus–dependent manner (12,16). These structural properties enable galectin-3 to bind several proteins, thus exerting multiple functions, which make it a broad-spectrum biologic response modifier (16) (Figure 1).

Intracellularly, galectin-3 acts as a pre-mRNA splicing factor (17). The nuclear splicing of pre-mRNA has been related to the co-localization in speckled structures with the Sm epitopes of the small nuclear ribonucleoprotein complexes and the non-small nuclear ribonucleoprotein splicing factor SC35 (18); the association/co-localization within the nucleus with the single-stranded DNA and with RNA, with the highest affinity to poly(A) ribonucleotide homopolymers (19); and the association with Gemin4 in survival of motor neuron–containing complexes, required for pre-mRNA splicing (20).

Galectin-3 also regulates the cell cycle, through a G1 or G2/M arrest, via downregulation of cyclins A and E, upregulation of p21 and p27 cyclin inhibitors, and hypophosphorylation of Rb protein (21). The cell-cycle regulating properties of galectin-3 may be a consequence of its fundamental action in RNA processing and include the control of both cell replication and death, possibly via several mechanisms. The relation of galectin-3 to cell proliferation is indicated by its upregulation and altered pattern of distribution and phosphorylation reported in 3T3 fibroblasts after mitogenic stimulation (22). Direct evidence of the mitogenic potential of galectin-3 has been obtained in human lung fibroblasts, that proliferated in response to galectin-3 in a dose-dependent, lactose-inhibitable manner (23), and human leukemia T-cell transfected with galectin-3 cDNA, showing higher growth rates than control transfectants (24). In addition to regulating cell proliferation, galectin-3 favors cell survival by protecting from apoptosis induced by a variety of death signals (24,25) and anoikis (21). Galectin-3 antiapoptotic activity seems to be related to the sequence homology (concerning the highly conserved NWGR

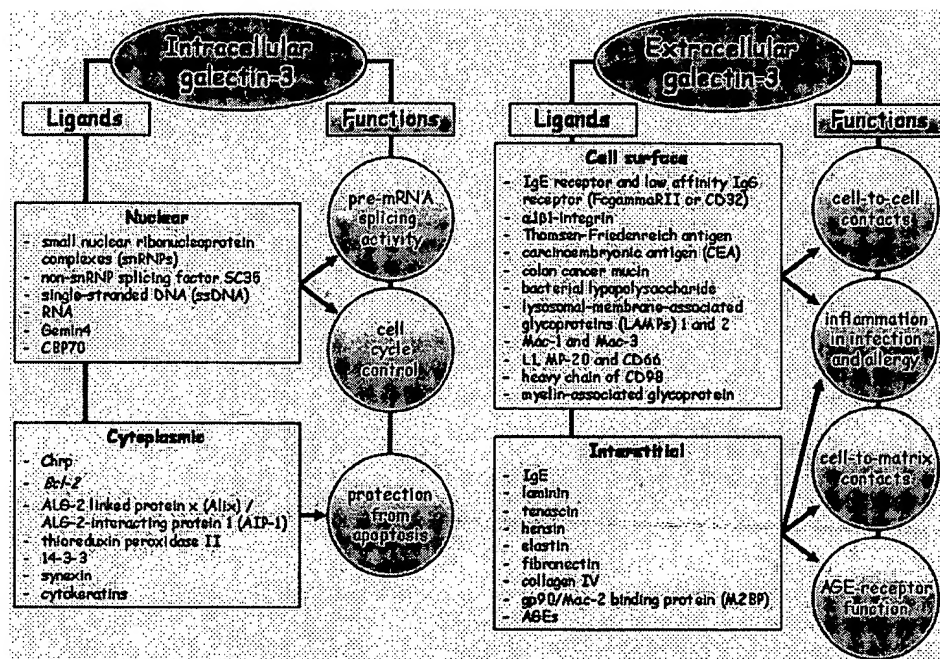


Figure 1. Ligands of intracellular and extracellular galectin-3 and related functions.

motif) and association with *bcl-2* (25) and the human homologue of ALG-2 linked protein x/ALG-2-interacting protein 1 (26). By virtue of its pro-proliferative and antiapoptotic action, galectin-3 is considered as an immediate early gene possibly implicated in tumor growth, as shown by the abnormal expression of galectin-3 reported in several neoplasms (27).

Extracellularly, galectin-3, regulates cell adhesion in a dual manner. Cell surface galectin-3 promotes homo- and heterotypic cell-to-cell interactions by serving as a cross-linking bridge between adjacent cells through attachment to a complementary serum glycoprotein (28). On the contrary, galectin-3 downregulates cell adhesion to the ECM component laminin via an association with the $\alpha 1 \beta 1$ -integrin receptor in a lactose-inhibitable manner, thus producing an antiadhesive effect (29). The increased interactions of tumor cells between each other and with the endothelium as well as the altered adhesion to the basement membrane glycoprotein laminin, resulting from this dual function on cell adhesion, are consistent features of the invasive and metastatic phenotype. Under certain circumstances, galectin-3 may promote cell adhesion to laminin, as shown in neutrophils (30). This effect is believed to play a role in the traversing of neutrophils through the basement membrane at the site of inflammation.

Another important function of extracellular galectin-3 is the modulation of inflammation, depending on its binding to IgE and the IgE receptor, that induces activation of mast cells (and basophils) (31), a central event in allergy. Eosinophils and neutrophils also bind IgE via galectin-3, which therefore participates in their IgE-dependent effector function (16). Finally, galectin-3 is capable of high-affinity binding, internalization, and degradation of AGE, thus playing a role in diabetes and aging.

Galectin-3 as an AGE-Receptor: Ligand Specificity, Interactions, and Function

Galectin-3 was found to exhibit high-affinity ^{125}I -AGE-BSA binding with saturable kinetics. Binding was fully blocked by excess unlabeled naturally formed or, under certain circumstances, synthetic AGE, but it was not inhibited or was weakly inhibited by either early glycation products or lectin-binding carbohydrates, such as lactose. Scatchard plot analysis was consistent with a single class of binding sites and an affinity similar to that of the AGE receptor on macrophages and higher than that for carbohydrates. Binding activity was retained by the C-terminal domain and even enhanced by removal of the N-terminal domain. Multimerization of galectin-3 onto the cell surface seems to be significant for displaying the AGE-receptor function, as indicated by the distinct patchy distribution assumed by galectin-3 when interacting with AGE (9).

The lack of transmembrane anchor sequence or signal peptide suggests that galectin-3 is associated with other AGE receptors rather than playing an independent role. In preliminary experiments from our group, total or cell membrane extracts of human mesangial cells were either immunoprecipitated with an anti-galectin-3 antibody or pulled down with a glutathione-S-transferase (GST) fusion protein with intact or C-terminal galectin-3. The precipitates reacted with either an anti-OST-48 or an anti-80K-H antibody, thus indicating that galectin-3 functionally interacts with both AGE-R1 and AGE-R2. This is consistent with the concept that these three molecules act as a molecular complex (the so-called "AGE-receptor complex") (9). In fact, AGE-R2 seems to have no or weak AGE-binding activity, because binding of AGE-modified proteins was not inhibited by anti-80K-H antibodies in most cells

(32,33). Conversely, it may be involved in signal transduction, as shown by its phosphorylation after exposure to AGE-BSA (33). In fact, the 80K-H protein was originally characterized as a protein kinase C substrate and later shown to be involved in signal transduction linking fibroblast growth factor-receptor 3 to mitogen-activated protein kinase (MAPK) (10). The implication of 80K-H in the activation of the p21(ras)-MAPK pathway suggests that AGE-R2 and the AGE-receptor complex could also participate in RAGE signaling. Moreover, galectin-3 interacts with gp90/Mac-2 binding protein (34), a multidomain glycoprotein forming linear and ring-shaped oligomers (35), whose domain 1 shows sequence homology with the cysteine-rich domain of the scavenger receptor (16). This suggests that the scavenger receptors also may interact with galectin-3 and the AGE-receptor complex.

In addition to binding AGE, galectin-3 recently was shown to play a role in endocytosis of AGE and modified (acetylated or oxidized) LDL (36), thus indicating ligand specificity and function similar to those of scavenger receptor; alternatively, it may activate this receptor to internalize AGE and LDL. In both cases, it seems to favor predominantly the removal of these damaging compounds.

The signaling events coupled with galectin-3 AGE-receptor function have not been elucidated. The co-localization within caveolin-rich membrane domains of the AGE-receptor complex with proteins involved in signal transduction and cytokine production, such as nitric oxide synthase, suggests a potential pathway for galectin-3 signaling (37).

Galectin-3 in Diabetic Nephropathy: Protection Toward AGE-Induced Renal Injury

Because of its multifunctional nature, galectin-3 could be involved in several neoplastic, inflammatory, and degenerative conditions. The demonstration of its ability to bind AGE has prompted the hypothesis that it also is implicated in vascular complications of diabetes, including nephropathy.

A few *in vivo* and *in vitro* studies have examined the expression of galectin-3 in vascular tissues under normal and pathologic conditions. In the vessel wall, smooth muscle cells did not express galectin-3, whereas endothelial cells showed low but detectable levels of this lectin. It is interesting that galectin-3 was induced in proliferating vascular smooth muscle cells as well as in smooth muscle and, particularly, foam cells from arteries of experimental animal models of atherogenesis and patients with advanced atherosclerotic lesions (38,39). In cultured endothelial cells, all of the components of the AGE-receptor complex were found to be present, with galectin-3 being weakly expressed under normal conditions and subjected to a marked upregulation in response to AGE (33). These findings indicate a possible participation of this lectin in the pathogenesis of diabetic (and nondiabetic) vascular disease. Galectin-3 was also found to induce endothelial cell morphogenesis and angiogenesis (40), a critical process in tumor growth and metastasis, but also in vascular complications. In the glomerulus, recent studies from our group have demon-

stration by the diabetic *milieu* (41). Galectin-3 protein and mRNA were not detectable in glomeruli from nondiabetic rats until 12 mo after initiating the study. On the contrary, in diabetic rats, galectin-3 expression was observed already at 2 mo of disease duration and increased thereafter. In cultured mesangial cells, no galectin-3 was detectable under normal glucose conditions (although it became evident after a certain number of passages in culture), whereas prolonged exposure to high glucose or addition of AGE induced or significantly increased galectin-3 expression.

In view of this galectin-3 (over)expression induced by the diabetic *milieu* in target tissues, we hypothesized that this lectin could participate in the pathogenesis of diabetic nephropathy by virtue of its AGE-receptor and growth-regulating properties, both potentially influencing glomerular remodeling. Therefore, we investigated the effects of galectin-3 deficiency on the development of experimental diabetic glomerulopathy, by using galectin-3 knockout mice, obtained by gene ablation, which were rendered diabetic with streptozotocin and killed 4 mo later, together with the corresponding wild-type animals (42). Despite a comparable degree of metabolic derangement, galectin-3-deficient mice developed accelerated glomerulopathy *versus* the wild-type animals, as evidenced by the significantly more pronounced increase in albuminuria, mesangial expansion, and the kidney cortex gene expression for the extracellular matrix components fibronectin, laminin, and collagen IV and the proinflammatory cytokine TGF- β . This was associated with a more marked renal/glomerular AGE accumulation, indicating that it was attributable to the lack of galectin-3 AGE-receptor function, impairing AGE uptake and degradation. The galectin-3-deficient genotype was associated with reduced expression of receptors implicated in AGE removal (macrophage scavenger receptor-A and AGE-R1) and increased mRNA expression of those mediating cell activation (RAGE and AGE-R2). Moreover, preliminary experiments in mesangial cells isolated from galectin-3 knockout and wild-type mice and either transfected with human galectin-3 cDNA or mock-transfected showed that the extent of matrix and TGF- β upregulation in response to high glucose and AGE is inversely related to the level of galectin-3 expression. The unchanged rates of glomerular cell proliferation and apoptosis detected in the knockout *versus* wild-type mice argued against the hypothesis that the cell cycle-regulating properties of galectin-3 were involved in the accelerated glomerulopathy occurring in these animals (42). This is at variance with other experimental rat models of glomerular and tubular disease, such as the acute mesangial proliferative glomerulonephritis induced by a single injection of anti-Thy1.1 antibodies (43) and the ischemia/reperfusion and folic acid-induced acute renal failure (44). In these settings, galectin-3 is upregulated and is considered to play a role in mesangial hypercellularity and tubular regeneration, respectively. Finally, we may not rule out the possibility that, in our study, the differential renal outcome in the two genotypes could be related, at least partly, to the lack of the adhesive and matrix-regulating properties of galectin-3.

Taken together, these observations suggest that galectin-3 acts as an AGE receptor to protect from AGE-induced tissue

injury. This protection might be provided either directly or through a modulation of the expression of the other AGE receptors to favor AGE degradation *versus* cell activation (Figure 2). Direct effects of galectin-3 could consist in an interference with RAGE signaling and downstream events and/or transduction of AGE signals through yet unknown intracellular pathways, possibly mediating AGE internalization and degradation. In addition, it may function as an extracellular ligand for AGE, just like soluble RAGE (see below; Figure 2).

These data also indicate that galectin-3 behaves differently from RAGE as an AGE receptor, thus supporting the concept that AGE receptors are functionally distinct. In fact, AGE binding to RAGE is associated with generation of reactive oxygen species triggering redox-sensitive signaling pathways. These pathways lead to p21^{ras}/MAPK-dependent activation of transcription factors such as nuclear factor κ B (NF- κ B) and the AP-1 complex (45,46) and the modulation of gene expression of several cytokines, with consequent induction of inflammation, coagulation, vasoconstriction, and adhesion (5). The predominant involvement of RAGE in AGE-induced cell activation and damage has been indicated by a recent report showing that mice that overexpress RAGE in vascular tissue and were cross-bred with another transgenic line that develops insulin-dependent diabetes shortly after birth, exhibited advanced diabetic nephropathy that was prevented by administration of the AGE inhibitor OPB-9195 (47). This is in keeping with observations showing that administration of soluble RAGE inhibited

the development of atherosclerosis in diabetic apolipoprotein E null mice (48) and arrested its progression when treatment was started after establishment of lesions (49).

Conclusions

The studies reviewed in this article showed that galectin-3 is upregulated in diabetes and aging, possibly as a result of increased AGE levels. Moreover, galectin-3-mediated AGE-receptor pathway is operating *in vivo* to confer protection toward AGE-induced tissue injury, as opposed to that through RAGE. This protection might be provided either directly or through the modulation of the expression of the other AGE receptors. As a future perspective, the functional specificity of AGE receptors could prompt the utilization of their pharmacologic or genetic manipulation to favor AGE degradation over cell activation and, hence, reduce the burden of carbonyl stress.

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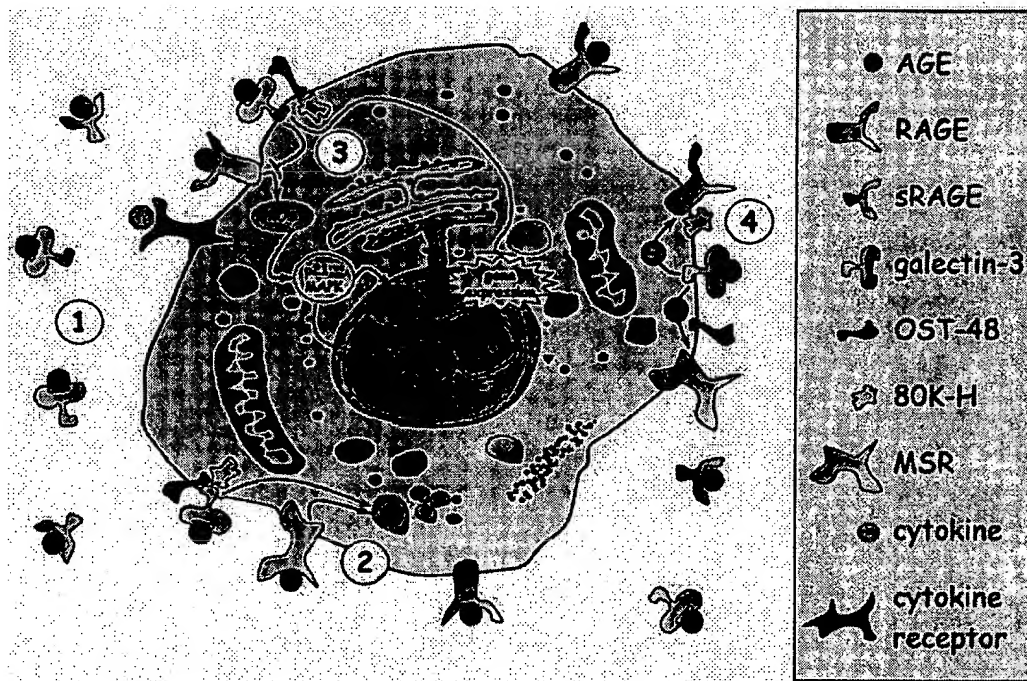


Figure 2. Mechanisms underlying galectin-3 protection toward AGE-induced injury. Protection could be provided via direct or indirect mechanisms. Direct effects include binding of AGE by secreted galectin-3, thus functioning like soluble RAGE (1); mediation of AGE internalization and removal by galectin-3 and/or the scavenger receptor (2); and interference with RAGE signaling and/or downstream events (3). The indirect mechanism consists in the modulation of the expression of the other AGE receptors by increasing those involved in AGE degradation (scavenger receptor and OST-48) and reducing those implicated in cell activation (RAGE and 80K-H; 4). ROS, reactive oxygen species; MSR, macrophage scavenger receptor; sRAGE, soluble RAGE.

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